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STOLBUR PHYTOPLASMAS IN TOMATOES

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16S r XII-A
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Hyalesthes obsoletus
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SUMMARY

Stolbur phytoplasma isolates belong to the subgroup 16S r XII-A, and are specialized bacteria that are obligate parasites of plant phloem tissue.

Stolbur phytoplasma has a broad plant host range which infects many crops of which more important for vegetable growing are tomato, potato and pepper.

The plant leafhopper *Hyalesthes obsoletus* play a key role in the spread of the disease. Phytoplasmas cause significant losses to farmers by different symptoms and diseases in more than 700 plant species.
This lead to dramatic changes in plant development, including witches broom, dwarfism, proliferation, phyllody, sterility of flowers, generalized yellowing and phloem necrosis.

We identified Stolbur phytoplasmas in three tomato cultivars grown in the region of Plovdiv.

Key words: stolbur, tomatoes

(16SrXII) , ,

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(Ember et al., 2011; Lee et al. 2000).

. Solanaceae – , ,

(Carraro et al., 2008; Navratil et al., 2009; Fialova et al., 2009, Ember et al., 2011).

1964 (, 1971).

(., 1964).

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(., 1970), (, 1974), (, 1977) (Avramov, 2008).

INTRODUCTION

Phytoplasma of the stolbur group (16SrXII) are phloem limited, insect-transmitted pathogens and they infect a great number of plants species. Depending on the affected species they induce various systemic symptoms ranging from yellowing, shoot proliferation, „witches-broom” and phyllody. Increasing incidence of stolbur phytoplasma was registered in different crops - grapevine, maize, sugar beet, potato, tomato and vegetable crops (Ember et al., 2011; Lee et al. 2000). In the vegetable crops, severe yield losses caused by stolbur phytoplasma have been recorded in Solanaceous crops - tomato, potato, pepper and celery (Carraro *et al*., 2008; Navratil et al., 2009; Fialova et al., 2009, Ember et al., 2011).

First report of phytoplasma diseases in Bulgaria was in 1964 (Kovachevski, 1971). Stolbur was reported on tomato and pepper (Kovachevski et al., 1964).

Virescence of strawberry petals was reported (Hristov et al., 1970), rose withering (Hristova, 1974), golden yellowing on the vine (Abrasheva, 1977), and stolbur of vine (Avramov, 2008).

With the improvement of methods for the identification of phytoplasma were identified new

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, Pear decline (Topchiiska et al., 2001), European fruit stone yellows phytoplasma (Topchiiska et al., 2002).

PCR

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(PCR).

Ahrens Seemuller (1992),

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(1 g) 4 ml CTAB (2% w/v

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1.4 M NaCl, 0.2% 2- -

, 20 mM EDTA, 100 mM Tris-HCl, 2%

, pH 8.0)

1.5 ml

65°C 30 .

-

(24:1) -

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PCR

phytoplasma, distributed on the territory of Bulgaria like Pear decline (Topchiiska et al., 2001), European fruit stone yellows phytoplasma (Topchiiska et al., 2002).

The present study demonstrates direct molecular identification of stolbur phytoplasma by PCR in tomato cultivars in Bulgaria.

MATERIAL AND METHODS

Plant Material. During the period April-May 2014 leaf samples from tomato cultivars Fantasy, Red Giant and Albeng's heart were collected from the region of Plovdiv. Samples consisted of leaves collected from plants showing typical symptoms of phytoplasma (Fig. 1, 2 and 3).

DNA Extraction and Polymerase Chain Reaction Amplification (PCR). DNA was extracted from fresh tomato leaves as described by Ahrens and Seemuller (1992), with some modifications. Tissue samples (1 g) were homogenized in 4 ml of CTAB buffer (2% w/v cetyltrimethylammonium bromide, 1.4 M NaCl, 0.2% 2- - mercaptoethanol, 20 mM EDTA, 100 mM Tris-HCl, 2% polyvinylpyrrolydone, pH 8.0) and 1.5 ml aliquots of the extract were incubated at 65°C for 30 min. DNA was further purified by phenol and chloroform-isoamyl alcohol (24:1) extraction and afterward precipitated. Eluted DNA template

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Nested PCR
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-
- ,
STOL11 -
720 bp,
(Stolbur
16SrXII) (Clair et al., 2003).

. PCR
STOL11f2 5 -
TATTTTCCTAAAATTGATTGGC-
3 STOL11r1 5 -
TGTTTTTGCACCGTTAAAGC-3 ,

- 830 bp.
PCR 22 µl
: PCR (10) –
2.2µl; – 16,7µl;
$MgCl_2$ (25 mM) – 0.35µl; (10
mM, 150 µ) – 0,3 µl.
STOL11f2/r1 -
0.0625µ (100µ) – 0.0125µl;
Taq 0.2U (15 U/µl) –
0.04 µl. BSA (20mg/ml) – 0.3µl;
– 2 µl.

(Auto Q Server QB-96 (LKB)):

92°C 3 min; 35
:
92°C 1 min,
55°C 1 min; 72°C
1,30 min;
72°C 10 min.

1:1000,

PCR.

was used for direct PCR amplification.

Nested PCR for identification of Stolbur phytoplasma, based on simultaneous amplification of non ribosomal DNA fragments, called STOL11 with length of 720 bp, which are specific for Stolbur group (Stolbur 16SrXII) (Clair et al., 2003).

The method is applied in two stages. The first PCR was performed with primers STOL11f2 5 - TATTTTCCTAAAATTGATTGGC-3 and STOL11r1 5 - TGTTTTTGCACCGTTAAAGC-3 , that flank the fragment length - 830 bp.

First stage of PCR in 22 µl total volume: PCR buffer (10x) – 2.2µl; distilled water – 16,7µl; $MgCl_2$ (25 mM) – 0.35µl; dNTPs (10 mM) – 0,3 µl. Primers STOL11f2 / r1 – 0.0625µM (100µM) – 0.0125µl; Taq polymerase 0.2U (15 U / µl) – 0.04 µl. BSA (20mg / ml) – 0.3µl; DNA extract – 2 µl.

Program of the thermocycler (Auto Q Server QB-96 (LKB)): first denaturation step at 92 ° C for 3 min; subsequent number of 35 cycles: denaturation at 92 ° C for 1 min, annealing at 55 ° C for 1 min; elongation at 72 ° C for 1,30 min; and a final elongation at 72 ° C for 10 min. After completion of the reaction, the amplified product is diluted 1: 1000 in order to use it in the subsequent second stage - nested PCR.

– Nested PCR
25μl:
– 16,48μl; – 150μ ,
(10mM,) – 0,3 μl;
STOL11f3/r2 - 0.375μ , (100μ)
– 0.075μl; PCR Taq – 1 ,
(10) – 2.5μl; $MgCl_2$ – 0.4mM (25 mM) – 0.38μl; BSA (0.2 mg mL–1)
– 0.3μl; Taq 0.6U (15 U/μl) – 0.04 μl.
5 μl
. nested PCR
STOL11f3
5 -ACGAGTTTTGATTATGTTCAC-3 STOL11r2 5 -GATGAATGATAACTTCAACTG-3,
720 bp.

nested PCR
PCR.
-
. 1,5%
0,5
110 V 20 min. 30 min
(1 μg/1 ml)
UV .
– 100 bp Ladder.

,
-
(Fig. 1, 2, 3)
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Second stage - Nested PCR in a total volume of 25μl: distilled water – 16,48μl; dNTP – 150μM, (10mM) – 0,3 μl; primers STOL11f3 / r2 – 0.375μM (100μM) – 0.075μl; PCR Taq buffer (10x) – 2.5μl; $MgCl_2$ – 0.4mM (25 mM) – 0.38μl; BSA (0.2 mg mL-1) – 0.3μl; Taq polymerase 0.6U (15 U / μl) – 0.04 μl. In the reaction mixture add 5 μl amplified and diluted product. For nested PCR we use primers STOL11f3 5’-ACGAGTTTTGATTATGTTCAC-3’i STOL11r2 5’–GATGAATGATAACTTCAACTG-3 that flank a fragment of 720 bp.

The reaction conditions for the nested PCR were the same as in the first PCR.

Electrophoresis and visualization. We used a 1.5% agarose gel in TAE buffer 0.5x electrolyte at a voltage of 110 V for 20 min. After 30 min incubation in a solution of ethidium bromide (1 μg/1 ml) of the DNA products in the agarose gel we made visualization with UV light. We used DNA marker – 100 bp ladder.

RESULTS AND DISCUSSION

Basic symptoms of stolbur which were observed in the analyzed three tomato cultivars were shoot proliferations (Fig. 1, 2, 3) and deformations of the leaf lamina, yellowing of some leaves and changing the color from green

to red and purple also.

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. 1. ( )

Fig. 1. Symptoms of Stolbur (shoot proliferations) on Tomato cv. Fantasy

.2. ( )

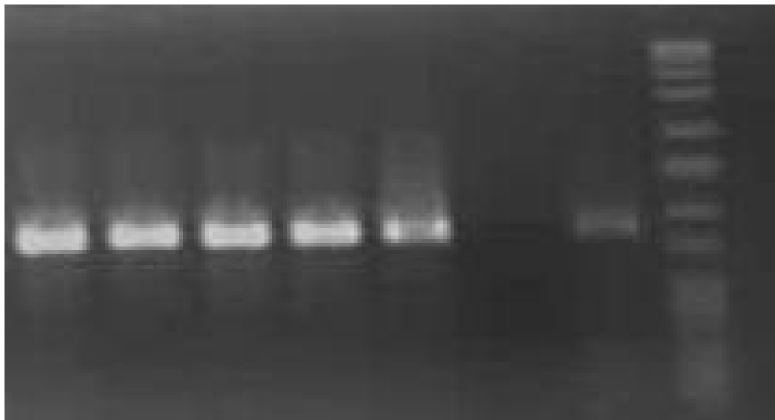
Fig.2. Symptoms of Stolbur (shoot proliferations) on Tomato cv. Red Giant

.3. ( )

Fig. 3. Symptoms of Stolbur (shoot proliferations) on Tomato cv. Albeng's heart

- - 6 24 , - nested-PCR 720 bp (Fig. 4). - , (.4). PCR .

The presence of phytoplasma was detected in 6 samples, out of 24 tested, symptomatic tomato plants (Fig. 1, 2, 3) resulting with an amplification of .720 bp DNA fragments using nested-PCR (Fig. 4). The positive samples were from the tomato cv. Fantasy, Red Giant and Albeng's heart (Fig.4). No visible PCR positive reactions were obtained from the other plants tested.



. 4. Nested PCR (
720 bp):
: 1,2 - ; 3,4 - ; 5,7 – , 6-
PCR mix, 8- (100bp DNA ladder, Sigma);

Fig.4. Nested PCR results of tomato samples (PCR fragment 720 bp):
Legend: 1,2 – Albeng's heart; 3,4 – Red giant; 5,7- Fantasy; 6 – PCR mix; 8 - 100bp DNA ladder, Sigma

- (,), nested-PCR. – .

CONCLUSIONS

Stolbur phytoplasmas was identified by direct nested PCR in three tomato cultivars (Red giant, Fantasy and Albeng's heart) in the region of Plovdiv. The three cultivars showed common symptoms of stolbur – shoot proliferations and leaf color changes.

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Research Institute of Mountain Stockbreeding and Agriculture, Troyan

PVY PLRV

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DISTRIBUTION AND CHARACTERIZATION OF PVY AND PLRV IN POTATO CULTIVAR AGRIA IN DIFFERENT REGIONS OF BULGARIA

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Y (PVY) (PLRV) -

SUMMARY

Potato virus Y (PVY) and Potato leaf roll virus (PLRV) are the most important viral pathogens of potato in Bulgaria. Different potato cultivars grown in Bulgaria react differently to the viral infection by these two pathogens under field conditions.

Some varieties are highly sensitive and others develop different degree of field resistance. Of particular importance are the regions of cultivation of potatoes. Both viruses are transmitted by vector – aphids, which have a major importance for their epidemiology.

We tracked the spread of these two viruses in the cultivar Agria in ten seed

. PVY ,
PLRV .
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: PVY, PLRV, Agria

(*Solanum tuberosum* L.) -
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-
-
(McDonald, 1984).
30
,
(Salazar, 1996).
Y (PVY,)
(, 2012),
-
(PLRV,), -
S (PVS,
),
X (PVX,)
M (PVM)
- -

regions of Bulgaria.
PVY occurs in five regions and PLRV occurs in three regions. Mixed infections from both viruses were observed in 2 regions, and caused the greatest losses for the producers. Only in three mountainous seed regions which were located at a high altitude were not detected viral infections.

Key words: PVY, PLRV, Agria

INTRODUCTION

Potato (*Solanum tuberosum* L.) is one of the most widely grown field crops in Bulgaria. To meet the demand of farmers, seed potatoes have been imported from other countries (mainly European), multiplied and then have been distributed to the private producers.

But, a vegetative propagated potato crop presents ample opportunity for the accumulation of viral pathogens with each multiplication in the field (McDonald, 1984).

here are more than 30 viral pathogens that can impact potato worldwide (Salazar, 1996).

Potato virus Y (PVY, a Potyvirus) (Petrov, 2012), Potat leaf roll virus (PLRV, a Polerovirus), Potato virus S (PVS, a Carlavirus), Potato virus X (PVX, a Potexvirus) and Potato virus M (PVM) are the most common viruses economically affecting potato crops in Bulgaria.

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(Singh, 1999).

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(Jones, 1988; Spiegel and Martin, 1993).

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– PVY PLRV

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These viruses can occur in single or as mixed infections within the potato crop (Singh, 1999).

In an effort to reduce extensive yield losses due to viral diseases in subsequent potato crops, seed tubers are tested prior to planting for the presence of viruses and the resultant virus-free tubers are planted (Jones, 1988; Spiegel and Martin, 1993).

The aim of the present study was to identify the most damaging viruses – PVY and PLRV in potatoes cv. Agria in different geographical regions in Bulgaria. In this way to find out the most appropriate places for virus free potato seed production.

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: DAS-ELISA (Double Antibody Sandwich nzyme Linked Immunosorbent Assay):

Clark Adams (1977).

LOEWE Biochemica GmbH, Sauerlach, Germany. ELISA - -

MATERIAL AND METHODS

Plant tuber material from 10 regions in Bulgaria– Bansko, Velingrad, Dragichevo, Kyustendil, Pazardjik, Pernik, Peshtera, Samokov, Smolyan and Trun.

Serological diagnostic test: DAS-ELISA (Double Antibody Sandwich nzyme Linked Immunosorbent Assay):

The analysis was conducted by the method of Clark and Adams (1977). We have used a commercial kit of LOEWE Biochemica GmbH, Sauerlach, Germany. ELISA plates are loaded

(IgG) PVY,
(
) 0.05 M
.
4 37 ° C,
PBS-T 5
.
,
1% PVP ( )
1:10.
4 °C 16 .
PVY
4
37 °C.
p- -
(p-nitrophenyl phosphate,
Sigma)
(pH 9.8) 1mg/ml.
3N NaOH.
-
-
(DTX 880)
405nm.

(OD) (Cut-off)
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10
. -
PVY,

with antiserum (IgG) for PVY, with dilutions (according to the instructions of the manufacturer) in 0.05 M carbonate buffer. The samples were incubated for 4 hours at 37°C, and the unbound components were washed out with PBS-T buffer for 5 min. All samples were grounded in extraction buffer containing 1% PVP (polyvinyl pyrrolidone) in a ratio of 1:10. The plates were incubated at 4 °C for 16 hours. Following the third wash step alkaline-phosphatase conjugate for PVY was added and the plates were incubated for 4 hours at 37 °C. The used substrate is p-nitrophenyl phosphate (p-nitrophenyl phosphate, Sigma) in diethanolamine buffer (pH 9.8) at a ratio of 1mg/ml. The reaction proceeded in the light at room temperature and was stopped with 3N NaOH. The adsorption of the color reaction is measured at multifunctional detector (DTX 880) at a wavelength of 405nm.

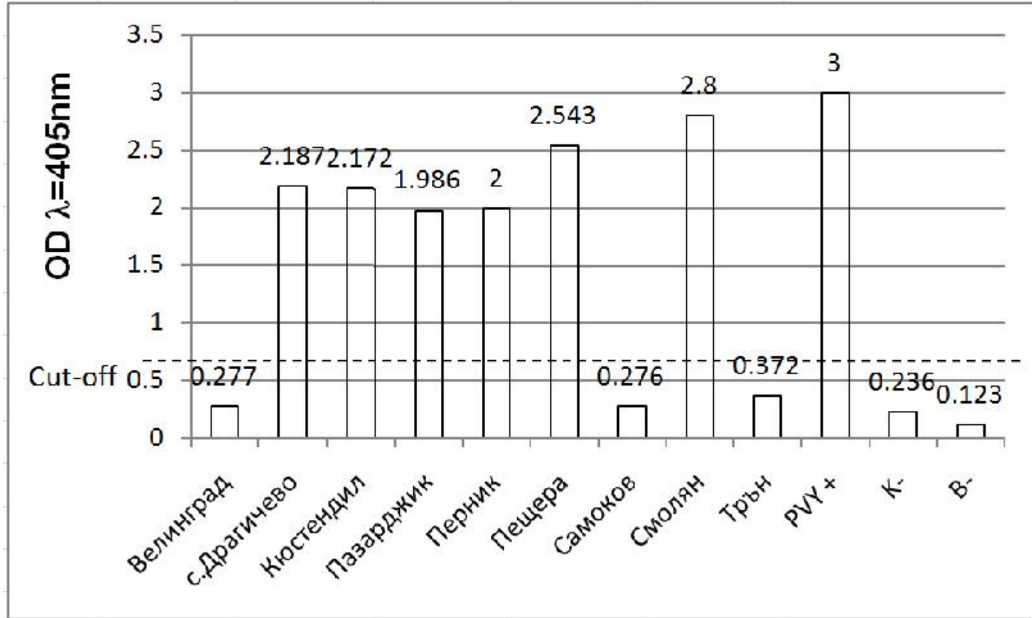
The positive samples had optical density (OD) over the threshold (Cut-off) which is three times the value of the negative control.

RESULTS AND DISCUSSION

From the analyzed potato tuber samples from the cv. Agria in 10 seed production regions of Bulgaria we received the following results. The most common virus is PVY, who was found in 6 seed

6 – , , - , , (. 1).

production regions – Dragichevo, Kyustendil, Pazardjik, Pernik, Smolyan and Peshtera (Fig. 1).



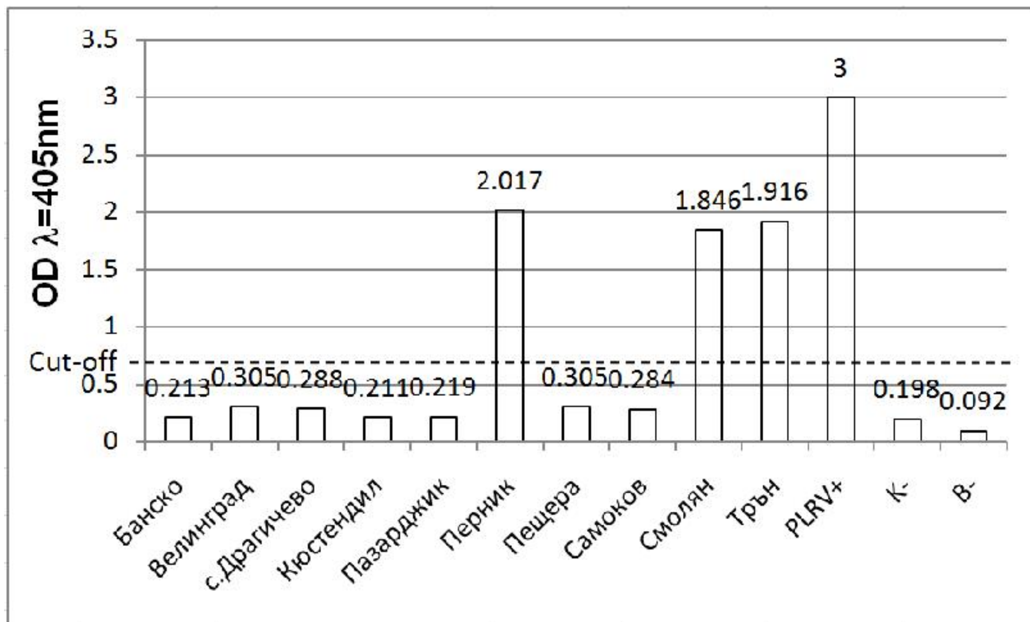
. 1. DAS-ELISA PVY

Fig. 1. DAS-ELISA results for PVY

. PLRV 3 – , (.2). – (.1; .2). - – , .

– .

Other geographical seed production regions lack this virus. PLRV was identified in 3 regions – Pernik, Smolyan and Trun (Fig.2). Mixed infections of the two viruses was identified in two regions – Smolyan and Pernik (Fig.1; Fig.2). Only in three geographical regions of Bulgaria viral infections in potato seed production tubers was not identified – Bansko, Velingrad and Samokov. These two geographical regions differ from the others by its relatively high altitude and terrain features and the corresponding microclimate prevent the spread of vectors of these viruses- aphids.



. 2. DAS-ELISA PLRV

Fig. 2. DAS-ELISA results for PLRV

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CONCLUSIONS

Mixed infections from both viruses were observed in 2 regions (Pernik and Smolyan), and caused the greatest losses for the producers. Only in three mountainous seed regions (Bansko, Velingrad and Samokov) which were located at a high altitude were not detected viral infections.

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